RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance

Louise Jones, Frank Ratcliff* and David C. Baulcombe

Background: The association between DNA methylation and gene silencing has long been recognized; however, signals that initiate de novo methylation are largely unknown. In plants, recognition of RNAs that are inducers of posttranscriptional gene silencing (PTGS) can result in sequence-specific DNA methylation, and the aim of this work was to investigate whether heritable epigenetic changes can occur by this mechanism and if the Met1 methyltransferase is required.

Results: RNA-directed DNA methylation (RdDM) was initiated in 35S-GFP transgenic plants following infection with plant RNA viruses modified to carry portions of either the 35S promoter or the GFP coding region. Targeting of the promoter sequence resulted in both methylation and transcriptional gene silencing (TGS) that was inherited independently of the RNA trigger. Targeting the coding region also resulted in methylation; however, this was not inherited. Expression of *Met1* was suppressed in order to investigate its role in initiation and maintenance of RdDM. Initiation of RdDM was found to be Met1-independent, whereas maintenance of methylation and TGS in the subsequent generations in the absence of the RNA trigger was Met1-dependent. Maintenance of methylation associated with systemic PTGS was also found to be Met1-independent.

Conclusions: RNA-triggered events can lead to heritable changes in gene expression, and it is possible that initiation of other epigenetic phenomena such as *trans*-silencing and paramutation may have an RNA component.

Address: Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK.

Correspondence: David C. Baulcombe
E-mail: david.baulcombe@bbsrc.ac.uk

*Present address: Syngenta Wheat Improvement Centre, John Innes Centre, Colney Lane, Norwich, NR4 7UH, UK.

Received: **1 March 2001**
Revised: **9 April 2001**
Accepted: **9 April 2001**

Published: **15 May 2001**

Current Biology 2001, 11:747–757

0960-9822/01/$ – see front matter

Background

Double-stranded (ds) RNA is a potent trigger of an RNA surveillance mechanism operating in a range of organisms [1–6]. This mechanism recognizes dsRNAs and processes them into small 21–25 nt RNAs [7–9] that are incorporated into an RNase complex [10]. The RNase complex can then target homologous RNAs for degradation. A second RNA-mediated, sequence-specific response has been identified in plants and is known as RNA-directed DNA methylation (RdDM) [11]. RdDM was first observed in tobacco plants carrying potato spindle tubor viroid (PSTVd) sequences as transgenes. Replication of the PSTVd RNA resulted in extensive methylation of the transgene DNA, but not of nonhomologous sequences elsewhere in the genome [12, 13]. RdDM has since been demonstrated using viral RNA [14–17] and has also been demonstrated for a transgene locus consisting of a transcribed inverted repeat [18]. Since viroids and viruses replicate via double-stranded intermediates and the inverted repeat transcript is capable of stem-loop formation, there is a correlation in each of the examples between the presence of double-stranded (ds) RNA and sequence-specific de novo methylation of the corresponding genomic region.

In two examples of RdDM, transgene silencing involved dsRNAs that are complementary to promoter regions. In the first example, Mette et al. [18] showed that nopaline synthase (nos) promoter-driven reporter genes could be *trans*-silenced by a transgene producing double-stranded nos promoter RNA. In an example from our laboratory, an RNA virus carrying a fragment of the 35S promoter was used to silence 35S-GFP transgenes [15]. In both systems, it was suggested, but not demonstrated directly, that the target genes had become silenced at the transcriptional level. The correlation between the inhibition of transcription and DNA methylation of promoter sequences is well known, and it is believed that the inhibitory effect is due to the promotion of localized changes in chromatin structure through the action of methyl-cytosine binding proteins and histone deacetylases [19, 20].

It has been proposed previously that the trigger for methylation associated with transcriptional gene silencing (TGS) is the DNA structure that may form at complex loci due to DNA-DNA interactions [21–24]. However, the recent evidence that TGS can be triggered by RNA challenges this conventional view and suggests that, for some examples of TGS, DNA methylation may have been triggered by RNA-DNA rather than DNA-DNA interactions.

If RdDM exists as a mechanism to induce epigenetic change, then it may be expected that such changes would be heritable. The aim of this work was to examine the consequences of RNA-triggered epigenetic changes and to assess the inheritance of these changes. We demonstrate that RNA-directed TGS, but not posttranscriptional gene silencing (PTGS), can be inherited independently of the RNA trigger and that inheritance correlates with maintenance of methylation. Using virus-induced gene silencing (VIGS) technology, by which host sequences carried in viral vectors inactivate the corresponding host mRNA, we have silenced the Met1 methyltransferase and show that inherited TGS and the associated methylation is lost. However, silencing of *Met1* affects neither methylation associated with maintenance of PTGS nor initiation of RNA-directed methylation. These results indicate that RdDM has a Met1-independent stage when the RNA trigger is present, whereas, in the subsequent generations and in the absence of the RNA trigger, maintenance of the methylation is Met1-dependent.

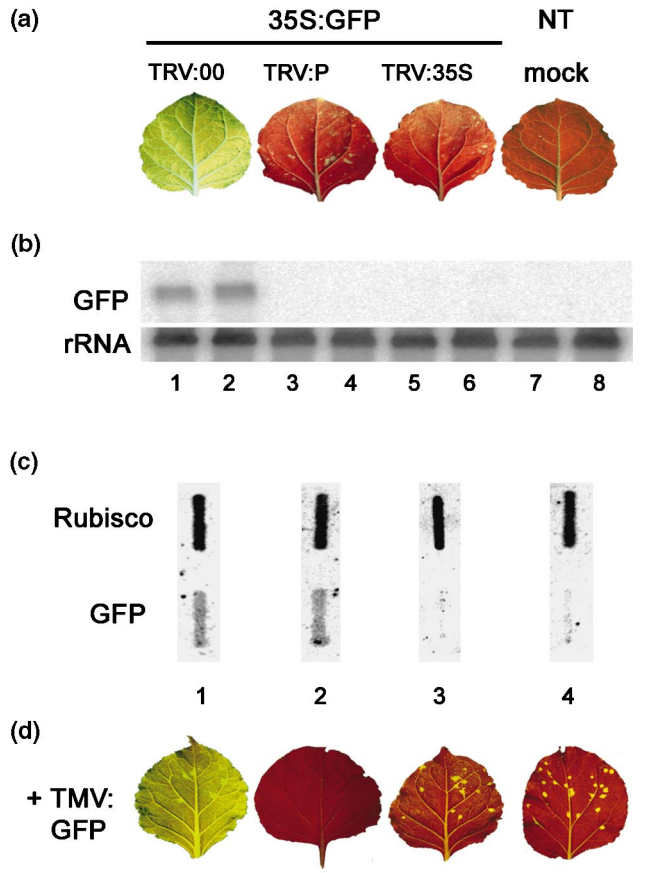
Results

Tobacco rattle virus (TRV) can induce posttranscriptional or transcriptional VIGS, depending on the region targeted

In this study, *Nicotiana benthamiana* line 16c or 8a plants carrying a single copy of a 35S-GFP transgene were infected with tobacco rattle virus (TRV) modified to carry the 3′ 359 nucleotides of GFP (TRV-P), 347 nucleotides of the 35S promoter sequence (TRV-35S), or TRV carrying no additional insert (TRV-00). From 11 days postinoculation (DPI), systemic infection of TRV-P and TRV-35S led to silencing of GFP (Figure 1a) manifested as loss of green fluorescence. Plants infected with TRV-00 showed no silencing of GFP. Northern blot analysis of GFP mRNA levels confirmed the visible silencing phenotypes (Figure 1b). These observations are in agreement with previous data using potato virus X (PVX) carrying 35S or GFP sequences [15, 25]. However, using TRV as a vector for virus-induced gene silencing (VIGS) has advantages over the use of PVX because it spreads more extensively than PVX into the growing points of infected plants [26].

Although we have established that silencing of GFP can be achieved by targeting either transcribed or nontranscribed portions of the 35S-GFP transgene, we had not determined whether the induced silencing was at the transcriptional or posttranscriptional level. To address this question, runoff transcription analyses were performed with nuclei isolated from nontransgenic *N. benthamiana* plants, 16c plants infected and silenced with TRV-P or TRV-35S, or 16c plants infected with TRV-00. Figure 1c shows that the ratio of transcription of the GFP transgene relative to that of the gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (rubisco) is similar in nuclei of TRV-00- (lane 1) and TRV-P-infected (lane 2) 16c plants. In nuclei of TRV-35S-infected plants (lane 3), very little GFP transcript is detected, and the hybridization profile is similar to that of nontransgenic nuclei (lane 4). Thus, the reduced GFP mRNA accumulation in TRV-P-infected plants is due to posttranscriptional

Figure 1



TRV can induce PTGS and TGS of a 35S-GFP transgene. **(a)** Systemic leaves of 35S-GFP plants that were infected with TRV-00, TRV-P, or TRV-35S, as indicated. A nontransgenic (NT) mock-inoculated leaf is shown as a comparison. Leaves were photographed at 14 DPI under UV illumination and are representative of at least ten independent experiments. **(b)** GFP mRNA levels in TRV-00- (lanes 1 and 2), TRV-P- (lanes 3 and 4), and TRV-35S- (lanes 5 and 6) infected 35S-GFP plants at 14 DPI and mock-inoculated nontransgenic plants (lanes 7 and 8). RNA extractions were performed in duplicate from separate plants and analyzed with a probe specific for the 5′ 400 bp of GFP. Five micrograms of total RNA were run per lane. The ethidium bromide-stained rRNAs are shown in the lower panel. **(c)** Nuclear runoff transcriptional analysis of TRV-00- (lane 1), TRV-P- (lane 2), and TRV-35S-infected (lane 3) 35S-GFP plants and mock-inoculated nontransgenic plants (lane 4). Nuclei were prepared from systemic leaves at 21 DPI, and ^{33}P-labeled nuclear RNA was used to probe slot filters containing PCR-generated fragments of either rubisco or GFP. Results are representative of three independent experiments. **(d)** GFP fluorescence in leaves of plants inoculated with TMV-GFP photographed under UV illumination. Plants were as described for (c), and results are typical of at least three independent experiments. Spots of GFP fluorescence correspond to foci of TMV-GFP infection.

gene silencing (PTGS), whereas for silencing induced by TRV-35S, reduction of GFP mRNA is at the level of transcription inhibition. These data were confirmed by infecting the plants with tobacco mosaic virus modified to express GFP (TMV-GFP) and by testing for RNA-based defense against incoming GFP sequences. From our previous work, we predicted that a GFP transgene silenced at the posttranscriptional level would confer resistance against TMV-GFP [27]. In contrast, a transgene silenced at the transcriptional level would not confer resistance. Figure 1d shows that TMV-GFP could accumulate on 16c plants silenced with TRV-35S (panel 3) and on control nonsilenced (panel 1) and nontransgenic plants (panel 4). Plants silenced with TRV-P (panel 2) were resistant to TMV-GFP, as predicted for tissue in which PTGS of GFP sequences has been activated.

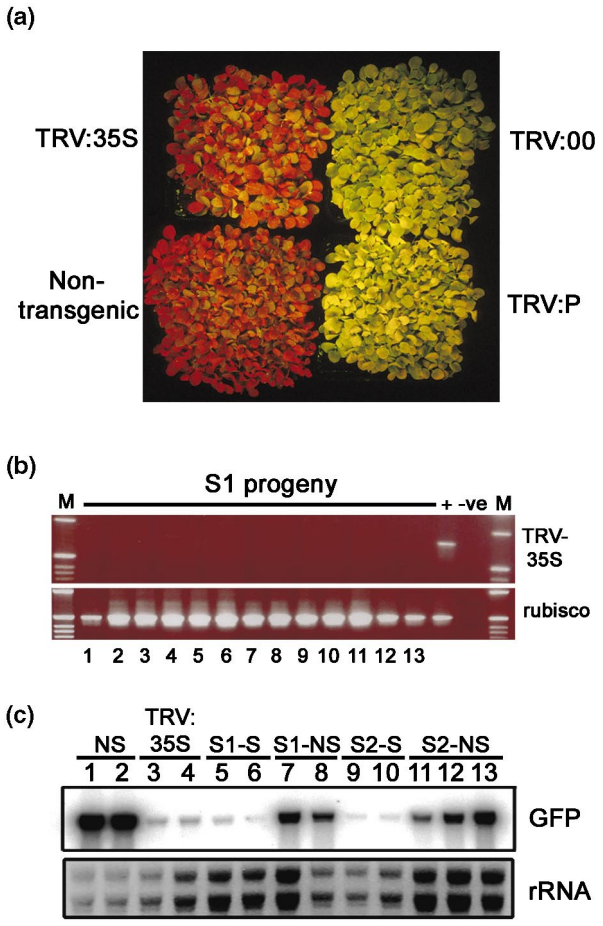
Virus-induced transcriptional, but not posttranscriptional, gene silencing is inherited

To determine whether silencing induced by the RNA-based mechanism of VIGS is heritable, we analyzed the progeny of TRV-P- and TRV-35S-silenced plants. Seed was collected from individual flowers that had been visually assessed for GFP silencing and also from control nonsilenced and nontransgenic plants. All of the progeny of TRV-P-silenced plants were green fluorescent to the same extent as progeny of TRV-00-infected plants, indicating that the PTGS induced by TRV-P is not inherited (Figure 2a). In contrast, the progeny of TRV-35S-silenced plants were red fluorescent, indicating that RNA-induced transcriptional silencing can be inherited (Figure 2a). These progeny were termed S1 for silenced generation 1. Thus, even though silencing of the 35S-GFP transgene can be triggered by an RNA-based mechanism using either TRV-P or TRV-35S, silencing is inherited differently.

It is unlikely that inheritance of TRV-35S-induced silencing was due to seed transmission of the virus, because rtPCR analysis on 13 representative progeny plants failed to detect TRV (Figure 2b). As a positive control for TRV infection, RNA was extracted from tissue of a TRV-35S-infected plant at 28 DPI. Virus levels in this tissue are low due to the natural process of recovery from TRV infection [26]. If TRV had been seed transmitted, we would expect virus levels equivalent or higher than those of the 28 DPI infected tissue. Figure 2b indicates that TRV-35S was not detectable in the S1 progeny plants (lanes 1–13), whereas it was detected in the sample from recovered tissue (lane 14). Amplification of rubisco cDNA in all test samples confirmed that cDNA was present in the samples used for rtPCR. Thus, we conclude that inheritance of transcriptional gene silencing is not due to the presence of TRV-35S in the progeny.

The young S1 progeny seedlings of TRV-35S-infected plants were red fluorescent. During the course of development, approximately 30% of these S1 plants remained fully silenced (termed S1-S), whereas the others (termed S1-NS) reverted to producing nonsilenced, green fluores-

Figure 2



RNA-triggered TGS is inherited. **(a)** Progeny of TRV-35S-, TRV-00-, and TRV-P-infected 35S-GFP transgenic plants. Progeny of a mock-inoculated nontransgenic plant are shown as a comparison. Seed were collected from individual flowers, sown in single pots, and photographed under UV illumination 1 week postgermination. Results are typical of seed from at least ten individual flowers per treatment. **(b)** Detection of TRV-35S and rubisco RNA by rtPCR in 13 S1 progeny (lanes 1–13) and a primary infected plant at 28 DPI (+). Lane 15 corresponds to a no-reverse transcriptase control reaction. The size of the rubisco amplification product corresponds to the cDNA sequence rather than to genomic DNA, indicating that cDNA synthesis had occurred. Amplification of the TRV sequence was only observed in samples from primary infected plants. **(c)** GFP mRNA levels in nonsilenced (NS, lanes 1 and 2) or TRV-35S-infected 16c plants (TRV-35S, lanes 3 and 4), S1 progeny that remained silenced (S1-S, lanes 5 and 6), S1 progeny that reverted to green fluorescence (S1-NS, lanes 7 and 8), S2 progeny that remained silenced (S2-S, lanes 9 and 10), and S2 progeny that reverted to green fluorescence (S2-NS, lanes 11, 12, and 13). RNA samples are from individual plants. Five micrograms of total RNA were run per lane and analyzed with a ^{32}P-labeled GFP specific probe. The ethidium bromide-stained rRNAs are shown in the lower panel.

cent leaves. The transition from silencing to nonsilencing was not associated with developmental sectors or sharp boundaries. The progeny of S1 plants (termed S2) were also assessed for GFP silencing. S1-S plants that maintained a fully silenced phenotype throughout development produced silenced S2 progeny. These S2 progeny were identical to the S1 generation in that some remained fully silenced, whereas others reverted to the nonsilenced state. The S2 progeny of S1s that had reverted to green fluorescence were likewise all green fluorescent. GFP mRNA levels were analyzed in these progeny plants by Northern blotting in order to confirm the visible phenotypes. S1-S (Figure 2c, lanes 5 and 6) and S2-S (lanes 9 and 10) progeny had GFP mRNA levels similar to the TRV-35S-infected parent plants (lanes 3 and 4). Progeny plants that had reverted to green fluorescence (S1-NS and S2-NS) had high GFP mRNA levels (Figure 2c, lanes 7, 8, 11, 12, and 13), similar to those of nonsilenced control plants (lanes 1 and 2).

To test the ability of a silenced 35S-GFP allele to *trans*-silence nonsilenced alleles, we carried out a series of crosses with silenced (S) and nonsilenced (NS) plants. TRV-35S-infected and -silenced 16c plants or silenced S1 progeny (S1-S) were crossed in a reciprocal manner with nonsilenced 16c plants. The progeny of five S1-S × NS crosses were all nonsilenced (data not shown). However, eight crosses using a primary infected plant as a parent all produced both silenced and revertant nonsilenced progeny (data not shown). The outcome was the same irrespective of whether the infected plant was the male or female parent. Similarly, in crosses between TRV-35S-infected 16c or S1-S plants and a nonallelic 35S-GFP transgenic line 8a, the *trans*-silencing was only obtained using a TRV-35S-infected plant as a parent (data not shown). These results indicate that *trans*-silencing requires a factor that is present in the TRV-35S-infected plants but absent in the S1 silenced progeny.

Inheritance of TGS is correlated with inheritance of methylation

The data in Figures 1 and 2 established that TGS can be induced by RNA and subsequently can be inherited independently of this trigger. Since there is often a strong correlation between methylation and TGS, we wished to determine whether methylation could be responsible for the inheritance of RNA-triggered TGS. Initially, we examined the methylation status of 35S and GFP sequences in DNA samples extracted from leaves and pollen of TRV-35S- and TRV-P-infected plants. DNA samples were analyzed by restriction enzyme digestion followed by real time quantitative PCR (Taqman, Applied Biosystems). This method allows the entire PCR reaction to be monitored, rather than just the end point, and therefore permits quantification to be based on the early linear part of the reaction. DNA was digested with Sau96I, a methylation-sensitive restriction enzyme that cuts within the 35S promoter and the GFP sequence. Methylation at these Sau96I sites will prevent digestion and therefore result in a higher level of amplifiable DNA compared to nonmethylated digested samples. Quantitative PCR was performed on two control sequences and on digested DNA using primer-probe combinations that spanned the Sau96I site in either the 35S promoter or GFP coding region. One control sequence did not contain Sau96I restriction sites and was used as a reference for quantification. The other control sequence spanned a Sau96I site but was not related to the 35S-GFP transgene. Amplification of this sequence in digested and undigested samples was used to determine whether each sample was digested to an equivalent extent.

Table 1 illustrates the amount of amplifiable 35S or GFP DNA following Sau96I digestion in DNA samples prepared from TRV-00-infected nonsilenced tissue and TRV-35S- or TRV-P-infected silenced tissue. The amplification values are obtained from the threshold cycle number (C_T), which is the cycle at which a significant increase in amplification is first detected. The C_T value is inversely proportional to the amount of amplifiable starting material, and thus amplification values are derived from the inverse log of the C_T value (Taqman PCR protocol, PE Applied Biosystems). In both leaves and pollen, the level of amplifiable DNA and therefore methylation in the 35S promoter of TRV-35S-infected plants was ~40–50 times greater than in DNA from nonsilenced TRV-00-infected tissue or TRV-P-infected tissue undergoing PTGS. GFP amplification, and hence methylation, was 23–70 times greater in samples from TRV-P- infected tissue than in samples from TRV-00- or TRV-35S-infected tissue. A similar pattern of amplification/methylation was also observed for another methylation-sensitive restriction enzyme, MaeII (data not shown). Thus, sequence-specific RNA-directed methylation can be detected in both leaves and pollen of the 35S promoter in plants infected with TRV-35S. Likewise, methylation of the GFP sequence can be detected in leaves and pollen of TRV-P-infected plants.

We then examined the methylation status of the 35S promoter and GFP sequences in the progeny of TRV-35S- and TRV-P-infected plants to determine whether methylation patterns detected in the primary infected plants are inherited. This analysis was performed using methylation-sensitive restriction enzyme digestion and gel-blot hybridization. For the 35S promoter, the restriction enzymes chosen were MaeII and HgaI, which have a symmetrical cytosine configuration in their recognition sequences, and Sau96I and XmnI, which contain cytosines in nonsymmetrical configurations.

Figure 3a shows that in samples prepared from tissue infected with TRV-35S (lanes 2, 5, 8, and 11), there were

Table 1

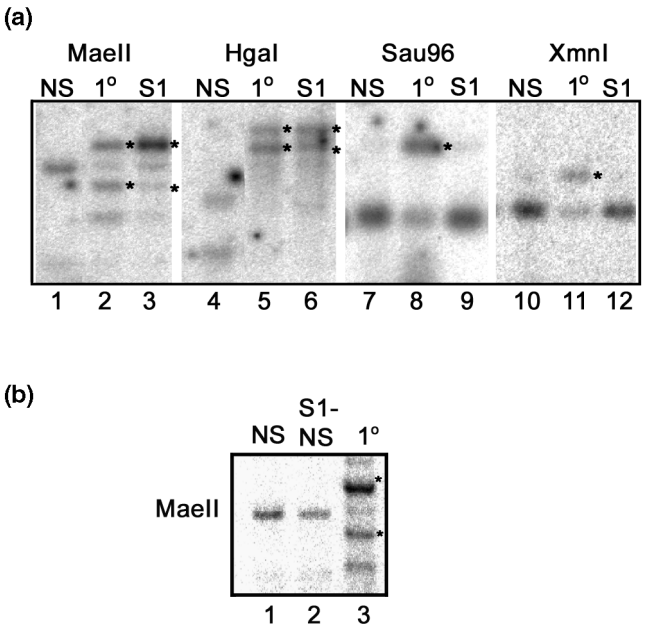
Detection of DNA methylation by restriction enzyme digestion and Taqman quantitative PCR.

	35S amplification values ± SD		GFP amplification values ± SD	
Treatment	Pollen	Leaves	Pollen	Leaves
TRV-00	0.056 ± 0.04	0.037 ± 0.21	0.35 ± 0.12	0.17 ± 0.07
TRV-35S	2.51 ± 0.92	1.97 ± 0.68	0.36 ± 0.21	0.3 ± 0.05
TRV-P	0.076 ± 0.19	0.042 ± 0.004	8.43 ± 4.5	11.95 ± 2.1

DNA samples were prepared from leaves and pollen of either TRV-00-, TRV-35S-, or TRV-P-infected 16c plants, digested with Sau96I, and analyzed by Taqman PCR with primers and probes spanning the Sau96I sites. Values represent levels of amplification obtained from the threshold cycle number, and there is a linear relationship between the amplification value and the amount of amplifiable starting material. Since cytosine methylation will inhibit Sau96I digestion, the higher the level of methylation, the higher the amplification value. Values are the average of at least three independent experiments, and the standard deviation (±SD) is shown. Values have been quantitatively standardized and take into account the level of digestion per sample.

35S-hybridizing fragments of higher molecular weight than those found in nonsilenced samples (lanes 1, 4, 7, and 10). These higher molecular weight bands can be accounted for by cytosine methylation within the enzyme recognition sequences. For tissue prepared from S1 plants, an identical hybridization pattern to that of the primary infected plants was observed for MaeII and HgaI digestions (compare lanes 2 and 5 with lanes 3 and 6), indicating that symmetrical methylation patterns are inherited.

Figure 3



Inheritance of RNA-directed DNA methylation. **(a)** DNA gel-blot analysis of samples from nonsilenced (NS; lanes 1, 4, 7, and 10), TRV-35S-infected (1°; lanes 2, 5, 8, and 11), and S1 progeny of TRV-35S-infected 35S-GFP transgenic plants (S1; lanes 3, 6, 9, and 12) and **(b)** DNA samples from nonsilenced (NS; lane 1), S1-NS progeny (S1-NS; lane 2), and TRV-35S-infected (1°; lane 3) plants. DNA samples were digested with MaeII, HgaI, Sau96I, or XmnI as indicated. The blot was probed with a probe that is specific for the 35S promoter. Fragments that can be accounted for by cytosine methylation within the enzyme recognition sequences are indicated by asterisks. Results are representative of three independent experiments.

In contrast, the 35S hybridization profile obtained for S1-S plants using Sau96I and XmnI (Figure 3a, lanes 9 and 12) was identical to that of nonsilenced plants (lanes 7 and 10). This observation suggests that the nonsymmetrical type of methylation in the primary infected plants may not be maintained in the next generation. Unfortunately, attempts at bisulphite sequencing for a more comprehensive methylation analysis proved to be unsuccessful, and therefore our conclusions are based solely on the available restriction enzyme sites.

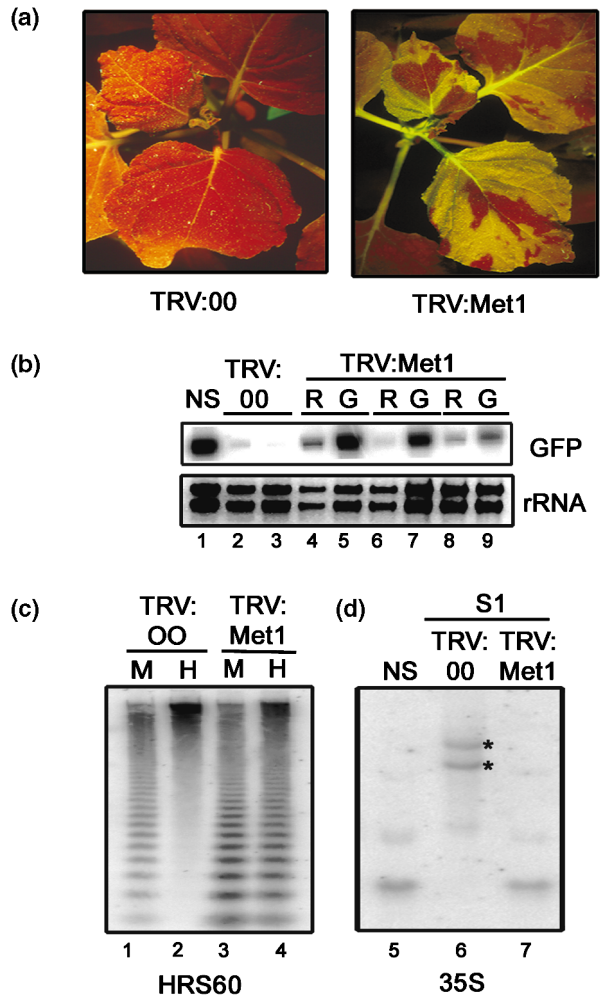
Figure 3b shows that S1 progeny that did not maintain TGS showed the nonsilenced 35S hybridization profile. For TRV-P-infected plants, GFP-specific DNA methylation was only observed in the primary infected plants, and neither symmetrical nor nonsymmetrical methylation was passed to the progeny (data not shown). Thus, for TGS, DNA methylation is inherited and correlates with silencing. For PTGS, although GFP-specific DNA methylation was detected in the pollen of primary infected plants, it is not present in the next generation.

VIGS of *N. benthamiana Met1*

To examine further the role of methylation in inheritance of RNA-triggered TGS, we used virus-induced gene silencing (VIGS) to suppress methyltransferase gene expression. The target methyltransferase was Met1, the enzyme that is thought to maintain methylation patterns [28]. A 180 bp fragment of the *N. benthamiana Met1* gene was generated by PCR using primers based on the tomato *Met1* sequence. This PCR fragment was cloned into the TRV vector, and the construct was used to infect silenced S1-S progeny.

At 11 DPI, reversal of TGS (Figure 4a) was clearly observed in TRV-Met1-infected plants. In silenced plants infected with TRV-00, no reversal was observed. RNA

Figure 4



Reversal of TGS by VIGS of *Met1* **(a)** Upper systemic leaves of transcriptionaly silenced S1-S 35S-GFP plants infected with either TRV-00 or TRV-Met1 as indicated. Plants were photographed at 21 DPI under UV illumination and are representative of at least 20 infected plants. **(b)** GFP mRNA levels in nonsilenced mock-inoculated 16c plants (NS; lane 1), TRV-00-infected S1-S TGS plants (TRV-00; lanes 2 and 3), red areas of TRV-Met1-infected S1-S TGS plants (lanes 4, 6, and 8), and green areas of TRV-Met1-infected S1-S TGS plants (lanes 5, 7, and 9). Five micrograms of total RNA were run per lane and analyzed with a GFP-specific probe. Ethidium bromide-stained rRNAs are shown in the lower panel. **(c)** DNA gel-blot analysis of TRV-00- (lanes 1 and 2) and TRV-Met1-infected (lanes 3 and 4) S1-S plants. DNA samples were prepared at 21 DPI and digested with either MspI or HpaII. The blot was hybridized with a probe that is specific for the HRS60 repeat. **(d)** DNA samples as for (c) and a sample from a nonsilenced (NS) 35S-GFP plant were digested with HgaI and hybridized with a 35S promoter-specific probe. Fragments that can be accounted for by cytosine methylation within the enzyme recognition sequence are indicated by asterisks.

was extracted from green and red fluorescent areas of individual leaves, and levels of GFP mRNA was analyzed by Northern blotting. Figure 4b shows that levels of GFP mRNA were higher in green fluorescent areas of TRV-Met1-infected plants (lanes 5, 7, and 9) than in red areas (lanes 4, 6, and 8), thus confirming the visible phenotype.

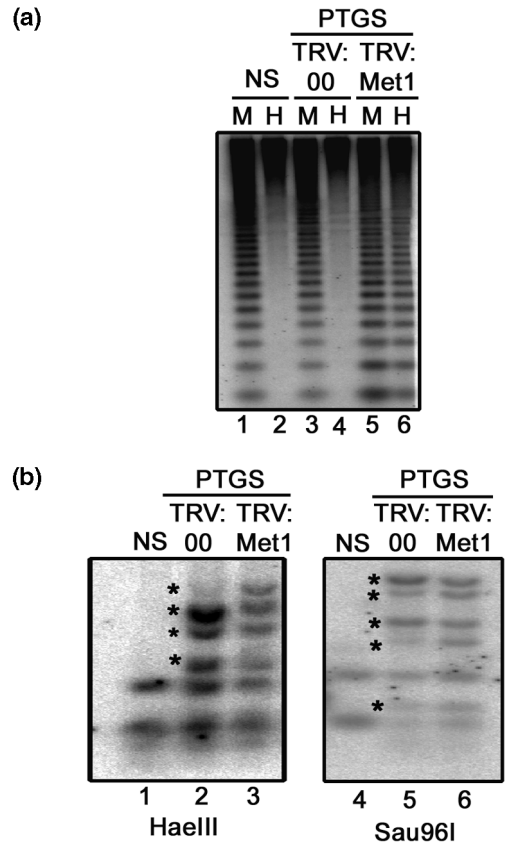
DNA was extracted from TRV-00 and the green fluorescent areas of TRV-Met1-infected S1 plants, and restriction enzyme digestion patterns of two sequences were analyzed in order to determine whether VIGS of *Met1* was affecting DNA methylation. The first sequence that was analyzed was a repetitive element homologous to the HRS60 family of repeats from *N. tabacum* [29]. In *N. tabacum*, this element is present in tandem arrays in the genome and is methylated. DNA was digested with isoschizomers, MspI or HpaII, and subjected to DNA gel-blot hybridization using the *N. tabacum* HRS60 monomeric repeat unit as a probe. For DNA samples from both TRV-00 and green fluorescent TRV-MetI-infected tissue, MspI digestion gave a ladder typical for tandemly arranged repetitive elements (Figure 4c, lanes 1 and 3). For HpaII digestion, most of the HRS60 DNA from TRV-00-infected plants was of a high molecular weight due to cytosine methylation within the HpaII recognition sequence (Figure 4c, lane 2). However, for DNA prepared from TRV-Met1-infected tissue, HpaII digestion gave a ladder pattern similar to that obtained for MspI digestion (Figure 4c, lane 4). Thus, TRV-Met1 infection causes hypomethylation of the HRS60 repeat, presumably due to VIGS of *Met1*. Methylation of the 35S promoter was assessed by digestion with HgaI followed by DNA gel-blot analysis. The 35S promoter that was used does not carry an HpaII site. Figure 4d shows that the higher molecular weight bands present due to methylation of HgaI recognition sites in the S1 plants (lane 6) are not present in S1 plants undergoing VIGS of *Met1* (lane 7). VIGS of *Met1* in S1 plants results in a 35S hybridization pattern identical to that observed in nonsilenced plants (lane 5).

Infection of S1-S plants with TRV-Met1 did not result in the complete reversion to the nonsilenced state (Figure 4a). In the areas that remained silenced, 35S DNA methylation was as high as in TRV-00-infected plants, and the HRS60 repeat was hypermethylated (data not shown). These observations indicate that VIGS of *Met1* was not active in these tissues, and it is likely, therefore, that the incomplete reversion to the nonsilenced state reflects the extent of infection of TRV-Met1 rather than the persistence of GFP silencing in the presence of VIGS of *Met1*. In tissue in which VIGS of Met1 was active (as assessed by HRS60 hypomethylation), the 35S promoter was hypomethylated and there was strong expression of GFP.

VIGS of *Met1* does not affect methylation associated with PTGS

The TRV-Met1 construct was also used to investigate whether Met1 has a role in maintaining patterns of methylation associated with PTGS of GFP. TRV-Met1 was inoculated to line 16c *N. benthamiana* plants in which

Figure 5



PTGS-specific methylation is not affected by VIGS of *Met1*. **(a)** DNA gel-blot analysis of the HRS60 repeat in DNA samples from nonsilenced 16c plants (lanes 1 and 2) and from plants undergoing PTGS of the 35S-GFP transgene that had been infected with either TRV-00 (lanes 3 and 4) or TRV-Met1 (lanes 5 and 6). DNA samples were prepared at 21 DPI, and results are representative of three independent experiments. DNA was digested with either MspI (M) or HpaII (H) as indicated. **(b)** DNA gel-blot analysis of the GFP coding region in DNA samples as described for 9a) and from nonsilenced 16c tissue (NS). DNAs were digested with either HaeIII or Sau96I as indicated and hybridized with a GFP-specific probe. DNA fragments that can be accounted for by cytosine methylation are indicated by asterisks.

systemic PTGS of GFP was established following localized introduction of 35S-GFP T-DNA [30]. We have demonstrated previously that systemic PTGS of the GFP transgene is associated with methylation [15]. After 20 DPI, no reversal of PTGS was observed, and the plants remained strongly red fluorescent (data not shown). DNA samples were prepared, and methylation of the HRS60 repeat and GFP sequences was analyzed by Southern blotting. Figure 5a confirms that the HRS60 repeat is hypomethylated in TRV-Met1-infected tissue. The enzymes used for GFP analysis were HaeIII and Sau96I. The HaeIII and Sau96I recognition sites are GGCC and GGNCC, respectively, and allow cytosines in both symmetrical (CG and CNG) and nonsymmetrical configurations to be analyzed, because guanine is adjacent to the enzyme recognition site in some, but not all, of the sites. Figure 5b shows that the GFP sequence remains as fully methylated in tissue undergoing VIGS of *Met1* (lanes 3 and 6) as it does in the TRV-00 control (lanes 2 and 5). Thus VIGS of *Met1* does not affect methylation associated with systemic PTGS.

In another set of experiments, the role of Met1 in the initiation of RdDM was addressed by coinoculating nonsilenced 35S-GFP transgenic plants with PVX-35S or PVX-P in combination with TRV-00 or TRV-Met1. We have shown previously that PVX-35S and PVX-P can induce silencing and RdDM of 35S-GFP transgenes [15], and therefore we tested if VIGS of *Met1* interferes with these processes. After 20 DPI, silencing of GFP initiated by PVX-35S or PVX-P was clearly visible in the newly emerging leaves. Analysis of the HRS60 repeat confirmed that, as shown previously, infection of TRV-Met1 results in hypomethylation of the repetitive elements (data not shown). Methylation of 35S or GFP sequences was analyzed by Southern blotting. The 35S and GFP sequences were found to be equally methylated in the presence of TRV-Met1 or TRV-00, indicating that VIGS of *Met1* does not affect initiation of RNA-directed methylation (data not shown).

Discussion

Met1 and RdDM

Our analysis with TRV-35S and TRV-P vectors indicates that RdDM involves two different mechanisms in the presence and absence of the RNA trigger. One of these mechanisms is manifested in the presence of the RNA trigger and is characterized by methylation of cytosines at symmetrical and nonsymmetrical configurations, as in other examples of RdDM [12, 31]. We can infer that Met1 is not required for this process, because VIGS of *Met1* had no affect on RdDM or the associated gene silencing with PVX-35S or PVX-P. The second mechanism was manifested in the progeny of TRV-35S-infected plants. DNA methylation and silencing persisted in these plants through several generations (Figures 2 and 3 and data not shown). The inherited methylation was restricted to cytosines in symmetrical contexts, and its maintenance was dependent on Met1 (Figure 4).

Our interpretation of these differences in sequence context and Met1 dependency is that there are distinct methyltransferases involved. One of these, active in the absence of the RNA trigger, is the product of *Met1*. However, in the presence of the RNA trigger, it seems likely that an RNA-directed DNA methyltransferase is required.

Several DNA methyltransferase sequences have been identified in plants that may be candidates for an RNA-directed DNA methyltransferase [32, 33]. Presumably, the dsRNA trigger of RdDM or the 21–25 nt species that are processed from the dsRNA [34] are involved in this process. Indeed, it may be informative to assess RdDM in mutants that are defective in recognition and processing of dsRNA to address whether it is the dsRNA or 21–25 nt RNAs that are directing methylation [35, 36]. These RNAs may affect the structure of homologous DNA so that it becomes a substrate for a de novo methyltransferase. Alternatively, the RNAs could associate with the DNA methyltransferase and guide the enzyme to its target DNA through base pairing interactions.

Maintenance of methylation

RdDM was not inherited when initiated by TRV-P, PVX-P, or PVX-35S or when triggered by transcription of an inverted repeat corresponding to the nos promoter [15, 18]. However, when RdDM was initiated by TRV-35S, it persisted through several generations. One explanation for the difference between heritable and nonheritable RdDM may derive from the presence of the initiator RNA in the meristem. If the methylation imprint is not established in meristematic cells, then it will not be carried through to the next generation. It seems likely that this meristem factor can account, at least in part, for the difference between the heritability of methylation induced by PVX-35S [15] and TRV-35S (Figures 2 and 3). TRV is able to access growing points and is known to induce silencing in these tissues, whereas PVX does not [26].

However, access to meristems cannot explain all aspects of heritability, because TRV-P-induced methylation persisted in the pollen (Table 1) but was absent in the progeny plants, whereas TRV-35S- induced methylation did persist (Table 1 and Figure 3). It is possible that the primary sequence, chromatin configuration, or transcriptional activity of the methylated region account for the observed differences. Similar factors may explain why inheritance of 35S methylation was not 100% stable in the S1 and subsequent generations.

It is unlikely that the RNA trigger that initiated RdDM in the primary infected plant was also present in the S1 and S2 generations since the virus was not present and there would have been no 35S RNA to maintain production of 35S dsRNA or 21–25 nt RNAs. Further indications that RNA mediators of silencing were absent include the susceptibility of S1 plants to reinfection with TRV-35S and our findings that silencing in S1 plants is not graft transmissible (L.J., unpublished data). Additionally, the patterns of DNA methylation and Met1 dependency were clearly different between the initially infected plants and S1 progeny.

Trans-silencing

A striking characteristic of TRV-35S-infected plants was the ability to silence and mediate RdDM of allelic and nonallelic sequences. This *trans*-silencing property was exhibited in crosses in which the infected plants were either parent but was not observed in the S1 and subsequent generations in which 35S methylation was maintained in the absence of the RNA trigger. It has been suggested previously that *trans*-silencing occurs by transient pairing of methylated and nonmethylated sequences and it is the complexity at the DNA level that is the signal for de novo methylation [23]. We cannot rule out the possibility that *trans*-silencing is related to the extensive symmetrical and nonsymmetrical cytosine methylation in the infected plants. However, we consider this possibility unlikely because the ability to carry out *trans*-silencing is associated with hallmarks of the RNA component of the silencing process. It seems more likely that the *trans*-silencing factor is an RNA.

There are many reports of *trans*-methylation and silencing, and the data presented here suggest that at least some of these are examples of RdDM. For example, in many aspects, our results are reminiscent of the well-studied epialleles of the *PAI* gene family in *Arabidopsis*. Characterization of epigenetic *pai* mutants demonstrated that an inverted *PAI* repeat is able to trigger methylation and silencing of unlinked homologous *PAI* sequences [37, 38]. Interestingly, transcription of the inverted repeat is required for *trans*-silencing, suggesting that an RNA component could be directing the methylation. Unlinked *PAI* loci become methylated on both symmetrical and nonsymmetrical cytosines in the presence of the inverted repeat locus and, when the repeat is removed, methylation is maintained almost exclusively on symmetrical cytosines. Given the similarity between the observations made for the *pai* epialleles and our data, it will be interesting to determine if an RNA surveillance mechanism is active against the *pai* inverted repeat and whether *pai* dsRNA or 21–25 nt small RNAs can be detected.

Other examples of silencing loci that are able to methylate unlinked loci with which they share sequence homology have been described. In some of these examples, the target locus does not lose methylation immediately after segregation away from the silencing locus and as such can maintain a heritable alteration in gene expression [39, 40]. In other examples, methylation is lost more rapidly [41]. Thus, these transgenic *trans*-silencing systems and the properties of the 35S-GFP transgene in TRV-35S- and TRV-P-infected plants resemble some naturally occurring examples of paramutation in which there are silencer and target loci that are methylated [42]. Like silencer trans-

genes, the paramutagenic loci are often repetitive loci, and there is variable persistence of the methylation and silencing of the target loci in the absence of the silencer [38, 43–45]. However, as shown here, the distinction between heritable and nonheritable methylation is not necessarily informative about the involvement of RdDM.

Since extensive nonsymmetrical cytosine methylation is associated with RdDM, it is tempting to use this as an indicator of RNA-DNA interactions. However, as we have demonstrated, in the absence of the RNA trigger, the nonsymmetrical methylation is lost. From this finding, we infer that there may be other examples of RNA-initiated silencing in which the methylation of nonsymmetrical cytosine residues is lost in the absence of the RNA trigger. However, we do not consider that all examples of epimutation can be attributed to RdDM. We consider, for example, that DNA-DNA interactions are the most likely explanation for the process of MIP (methylation induced premeiotically) in *Asocobolus*, in which a correlation between silencing, methylation, and homologous recombination has been observed [46]. Furthermore, the epialleles of *AGAMOUS* and *SUPERMAN* in *Arabidopsis* are heavily methylated, and it is difficult to attribute an RNA trigger for methylation in these examples [47, 48]. Both of these genes are associated with pyrimidine-rich sequences that may form secondary structures that are particularly susceptible to methylation.

Methylation and PTGS

There are conflicting data concerning the role of DNA methylation in PTGS. In some examples, there is no correlation between PTGS and DNA methylation [52–54]. However, in other systems, the correlation is strong [14, 49–51]. In one of these examples, the PTGS and the associated methylation of a GUS transgene were reversed in *met1* mutant *Arabidopsis* plants [50]. With the examples of induced PTGS described here, the situation is apparently intermediate, because there is a strong association of PTGS with transgene methylation, but from the VIGS experiment, there is no requirement of Met1 for maintenance of the methylation (Figure 5).

From these observations, it seems that the association of DNA methylation and PTGS is preserved in *Met1*-dependent and *Met1*-independent systems of maintenance. In a *Met1*-dependent system, there is apparently a causal relationship of transgene methylation and PTGS [50]. It will be interesting to find out whether the same relationship applies in *Met1*-independent systems of PTGS. One approach to this question may be to use VIGS to suppress different DNA methyltransferases in order to determine which enzyme is responsible for RdDM and whether inhibiting such methylation affects PTGS. VIGS could also be used in a forward genetics approach to search for genes involved in PTGS or TGS [55].

Materials and methods

Plant material

Transgenic *Nicotiana benthamiana* lines 16c and 8a carrying single 35S-GFP transgenes were described previously [25].

Wild-type and recombinant viruses

TRV has a bipartite genome. The construction of infectious binary vector clones of RNA1 (pTV00) and RNA2 (pBINTRA6) has been described previously [26]. TRV-P, TRV-35S, and TRV-Met1 were all constructed by inserting fragments into the SmaI site of pTV00. TRV-P carries a 321 bp fragment corresponding to the 3′ end of GFP [26]. TRV-35S carries a 347 bp Asp718-HindIII fragment of the 35S promoter from pJIT121. TRV-Met1 carries a 180 bp fragment of the *N. benthamiana Met1* gene that was PCR amplified from *N. benthamiana* cDNA using primers 5′-GGG TTTTCTGGAATGAA-3′ and 5′-ACAAAATTCCTAACATTCTC-3′. These primers were based on the tomato *Met1* sequence (accession number AJ002140), although recently, the *N. tabacum* sequence is available (accession number AB030726) [56]. Sequencing of the PCR fragment confirmed that it was the *N. benthamiana Met1* homolog and it is 99% and 95% identical to the *N. tabacum* and tomato *Met1* sequences, respectively. To generate a TRV infection, *Agrobacterium*-mediated transient gene expression of infectious constructs from the T-DNA of binary plasmids pTV00 and pBINTRA6 was used as described previously [26].

PVX-P, PVX-35S, and TMV-GFP have been described previously [15, 57].

Nuclear runoff transcription analyses

Nuclei for runoff transcription analyses were isolated as described previously [58]. Incorporation of uridine 5′-^{33}P-triphosphate was determined by probing 1 μg of the appropriate denatured PCR fragment immobilized onto Hybond N+ membranes (Amersham). Incorporation was assessed using Fujix Bio-imaging analyzer Bas 1000 equipment (Fuji Photo Film).

DNA extraction and gel-blot analysis

Genomic DNA was extracted from leaves and pollen using the DNeasy plant DNA extraction kit (Qiagen) according to manufacturer's instructions. DNA gel-blot analysis was performed as described previously [14]. ^{32}P-labeled probes corresponded to the entire 812 bp of GFP, the 347 bp Asp718-HindIII fragment of the 35S promoter, or the 182 bp HRS60 repeat of *N. tabacum* [29].

RNA extraction and gel-blot analysis

Total RNA was extracted using Tri-reagent (Sigma) according to the manufacturer's instructions. RNA gel electrophoresis and gel-blot analysis were performed as described previously [14].

Methylation analysis by Taqman quantitative PCR

DNA was extracted from leaves and pollen using the DNeasy plant DNA extraction kit (Qiagen). Pollen was collected from ten flowers for each extraction. Approximately 10 ng of genomic DNA was digested overnight with Sau96 or MaeII in a total volume of 100 μl. Enzyme activity was then terminated by heating at 65°C for 15 min, and the samples were diluted 2-fold in distilled water. Control undigested samples were treated in the same way, but the enzymes were not included in the reaction. Quantitative PCR was performed using an ABI Prism 7700 sequence detection system with 2× Taqman Universal PCR master mix (PE Applied Biosystems). The PCR reactions were performed in triplicate for each sample. Real time amplification plots were used to determine the threshold cycle number (C_T), which is the cycle at which a significant increase in amplification (as measured by release of the fluorescent dye from the probe) is first detected (Taqman PCR protocol, PE Applied Biosystems).

For GFP amplification, the primer sequences were 5′-CCTGTCCTTT TACCAGACAACCA-3′ and 5′-CCCAGCAGCTGTTACAAACTCA-3′, and the probe sequence was 5′-ACCTGTCCACACAATCTGCCCT TTCG-3′. For 35S amplification, the primer sequences were 5′-GCC GACAGTGGTCCCAAA-3′ and 5′-CCTTACGTCAGTGGAGATATCA CATC-3′, and the probe sequence was 5′-TCCAACACGTCTTCA AAGCAAGTGGA-3′. Each sample was quantitatively standardized by amplification of a sequence that did not span a Sau96I or MaeII site using primers 5′-ACCACAGGGATAACTGGCTTGT-3′ and 5′-CCGA CATCGAAGGATCAAAAA-3′ and probe 5′-CAGCCAAGCGTTCATA GCGACGTT-3′. Levels of digestion for each sample were analyzed by comparison of amplification of undigested and digested DNA using primers 5′-AGGTTTTCTGCGTTCAGTCAATC-3′ and 5′-CGGACGG CAGCCCTTT-3′ and probe 5′-AGAGTGAATCGGTCCCTAAGGAA CCCCC-3′. For all DNA samples, levels of digestion were found to be approximately equal. All probe DNA was modified to contain 5′-FAM fluorescent reporter and 3′-Tamra quencher (MWG).

Acknowledgements

We are grateful to the Gatsby Charitable Foundation for supporting the work in the Sainsbury Laboratory. We are thankful to our colleagues in the Sainsbury Laboratory for discussions. The work was carried out under license from the Ministry of Agriculture, Fisheries and Food (PHL 24B/3654).

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.** *Nature* 1998, **391:**806-811.
2. Waterhouse PM, Graham HW, Wang MB: **Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA.** *Proc Natl Acad Sci USA* 1998, **95:**13959-13964.
3. Chuang CH, Meyerowitz EM: **Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*.** *Proc Natl Acad Sci USA* 2000, **97:**4985-4990.
4. Kennerdell JR, Carthew RW: **Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway.** *Cell* 1998, **95:**1017-1026.
5. Wianny F, Zernicka-Goetz M: **Specific interference with gene function by double-stranded RNA in early mouse development.** *Nat Cell Biol* 2000, **2:**70-75.
6. Wargelius A, Ellingsen S, Fjose A: **Double-stranded RNA induces specific developmental defects in zebrafish embryos**. *Biochem Biophys Res Commun* 1999, **263:**156-161.
7. Hamilton AJ, Baulcombe DC: **A novel species of small antisense RNA in post-transcriptional gene silencing.** *Science* 1999, **286:**950-952.
8. Bernstein E, Caudy AA, Hammond SM, Hannon GJ: **Dicer, and RNaseIII-family nuclease, executes the initiation step of RNAi.** *Nature* 2001, **409:**363-366.
9. Zamore PD, Tuschl T, Sharp PA, Bartel DP: **RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals.** *Cell* 2000, **101:**25-33.
10. Hammond SM, Bernstein E, Beach D, Hannon G: **An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cell extracts.** *Nature* 2000, **404:**293-296.
11. Wassenegger M: **RNA-directed DNA methylation.** *Plant Mol Biol* 2000, **43:**203-220.
12. Wassenegger M, Heimes S, Riedel L, Sänger HL: **RNA-directed de novo methylation of genomic sequences in plants.** *Cell* 1994, **76:**567-576.
13. Pelissier T, Thalmeir S, Kempe D, Sanger HL, Wassenegger M: **Heavy de novo methylation at symmetrical and nonsymmetrical sites is a hallmark of RNA-directed DNA methylation.** *Nucleic Acids Res* 1999, **27:**1625-1634.
14. Jones AL, Thomas CL, Maule AJ: **De novo methylation and cosuppression induced by a cytoplasmically replicating plant RNA virus.** *EMBO J* 1998, **17:**6385-6393.
15. Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC: **RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing**. *Plant Cell* 1999, **11:**2291-2302.
16. Guo HS, Lopez-Moya JJ, Garcia JA: **Mitotic stability of infection-induced resistance to plum pox potyvirus associated with transgene silencing and DNA methylation.** *Mol Plant-Microbe Interact* 1999, **12:**103-111.
17. Thomas CL, Jones L, Baulcombe DC, Maule AJ: **Size constraints for targeting post-transcriptional gene silencing and for RNA-directed methylation in *Nicotiana benthamiana* using a potato virus X vector.** *Plant J* 2001, **25:**1-11.
18. Mette MF, vanderWinden J, Matzke MA, Matzke AJM: **Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans.** *EMBO J* 1999, **18:**241-248.
19. Ng HH, Bird A: **DNA methylation and chromatin modification**. *Curr Opin Genet Dev* 1999, **9:**158-163.
20. Kass SU, Pruss D, Wolffe AP: **How does DNA methylation repress transcription?** *Trans Genet* 1997, **13:**444-449.
21. Matzke MA, Matzke AJM: **How and why do plants inactivate homologous (trans)genes?** *Plant Physiol* 1995, **107:**6679-6685.
22. Bender J: **Cytosine methylation of repeated sequences in eukaryotes: the role of DNA pairing.** *Trends Biochem Sci* 1998, **23:**252-256.
23. Muskens MWM, Vissers APA, Mol JNM, Kooter JM: **Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing.** *Plant Mol Biol* 2000, **43:**243-260.
24. Matzke MA, Matzke AJM, Eggleston WB: **Paramutation and transgene silencing: a common response to invasive DNA?** *Trends Plant Sci* 1996, **1:**382-388.
25. Ruiz MT, Voinnet O, Baulcombe DC: **Initiation and maintenance of virus-induced gene silencing**. *Plant Cell* 1998, **10:**937-946.
26. Ratcliff F, Martin-Hernandez AM, Baulcombe DC: **Tobacco rattle virus as a vector for analysis of gene function by silencing.** *Plant J* 2001, **25:**237-245.
27. Ratcliff F, MacFarlane S, Baulcombe DC: **Gene silencing without DNA: RNA-mediated cross protection between viruses.** *Plant Cell* 1999, **11:**1207-1215.
28. Finnegan EJ, Kovac KA: **Plant DNA methyltransferases**. *Plant Mol Biol* 2000, **43:**189-201.
29. Vyskot B, Koukalova B, Kovarik A, Sachambula L, Reynolds D, Bezdek M: **Meiotic transmission of a hypomethylated repetitive DNA family in tobacco**. *Theor Appl Genet* 1995, **91:**659-664.
30. Voinnet O, Baulcombe DC: **Systemic signalling in gene silencing**. *Nature* 1997, **389:**553.
31. Wang MB, Wesley SV, Finnegan EJ, Smith NA, Waterhouse PM: **Replicating satellite RNA induces sequence-specific DNA methylation and truncated transcripts in plants. *RNA-Publ*** *RNA Soc* 2001, **7:**16-28.
32. Genger RK, Kovac KA, Dennis ES, Peacock WJ, Finnegan EJ: **Multiple DNA methyltransferase genes in *Arabidopsis thaliana***. *Plant Mol Biol* 1999, **41:**269-278.
33. Cao X, Springer N, Muszynski MG, Phillips RL, Kaeppler S, Jacobsen S: **Conserved plant genes with similarity to mammalian *de novo* DNA methyltransferases**. *Proc Natl Acad Sci USA* 2000, **97:**4979-4984.
34. Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJM: **Transcriptional silencing and promoter methylation triggered by double-stranded RNA**. *EMBO J* 2000, **19:**5194-5201.
35. Dalmay T, Hamilton AJ, Rudd S, Angell S, Baulcombe DC: **An RNA-dependent RNA polymerase gene in *Arabidopsis* is required for posttranscriptional gene silencing mediated by a transgene but not by a virus.** *Cell* 2000, **101:**543-553.
36. Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel J-B, *et al.*: ***Arabidopsis SGS2* and *SGS3* genes are required for posttranscriptional gene silencing and natural virus resistance.** *Cell* 2000, **101:**533-542.
37. Bender J, Fink GR: **Epigenetic control of an endogenous gene family is revealed by a novel blue fluorescent mutant of *Arabidopsis*.** *Cell* 1995, **83:**725-734.
38. Luff B, Pawlowski L, J. B: **An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*.** *Mol Cell* 1999, **3:**505-511.
39. Park YD, Papp I, Moscone EA, Iglesias VA, Vaucheret H, Matzke AJM, *et al.*: **Gene silencing mediated by promoter homology occurs at the level of transcription and results in meiotically heritable alterations in methylation and gene activity.** *Plant J* 1996, **9:**183-194.
40. Vaucheret H: **Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of**

homology in the promoter sequence are sufficient for trans-inactivation. *C R Acad Sci Paris* 1993, **316:**1471-1483.
41. Jakowitsch J, Papp I, Moscone EA, van der Winden J, Matzke M, Matzke AJM: **Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters in *trans*.** *Plant J* 1999, **17:**131-140.
42. Hollick JB, Dorweiler JE, Chandler VL: **Paramutation and related allelic interactions**. *Trans Genet* 1997, **13:**302-308.
43. Bollmann J, Carpenter R, Coen ES: **Allelic interactions at the *nivea* locus of antirrhinum.** *Plant Cell* 1991, **3:**1327-1336.
44. Walker EL: **Paramutation of the r1 locus of maize is associated with increased cytosine methylation.** *Genetics* 1998, **148:**1973-1981.
45. Stam M, Viterbo A, Mol JNM, Kooter JM: **Position-dependent methylation and transcriptional silencing of transgenes in inverted T-DNA repeats: implications for posttranscriptional silencing of homologous host genes in plants.** *Mol Cell Biol* 1998, **18:**6165-6177.
46. Colot V, Maloisel L, Rossignol JL: **Interchromosomal transfer of epigenetic states in Ascobolus: transfer of DNA methylation is mechanistically related to homologous recombination.** *Cell* 1996, **86:**855-864.
47. Jacobsen SE, Sakai H, Finnegan EJ, Cao X, Meyerowitz EM: **Ectopic hypermethylation of flower-specific genes in *Arabidopsis***. *Curr Biol* 2000, **10:**179-186.
48. Jacobsen SE, Meyerowitz EM: **Hypermethylated SUPERMAN epigenetic alleles in *Arabidopsis*.** *Science* 1997, **277:**1100-1103.
49. Ingelbrecht I, Van Houdt H, Van Montagu M, Depicker A: **Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation.** *Proc Natl Acad Sci USA* 1994, **91:**10502-10506.
50. Morel JB, Mourrain P, Beclin C, Vaucheret H: **DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*.** *Curr Biol* 2000, **10:**1591-1594.
51. English JJ, Mueller E, Baulcombe DC: **Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes.** *Plant Cell* 1996, **8:**179-188.
52. Van Blokland R, Van der Geest N, Mol JNM, Kooter JM: **Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover.** *Plant J* 1994, **6:**861-877.
53. Goodwin J, Chapman K, Swaney S, Parks TD, Wernsman EA, Dougherty WG: **Genetic and biochemical dissection of transgenic RNA-mediated virus resistance.** *Plant Cell* 1996, **8:**95-105.
54. Sonoda S, Nishiguchi M: **Graft transmission of post-transcriptional gene silencing: target specificity for RNA degradation is transmissible between silenced and non-silenced plants, but not between silenced plants.** *Plant J* 2000, **21:**1-8.
55. Baulcombe DC: **Fast forward genetics based on virus-induced gene silencing.** *Curr Opin Plant Biol* 1999, **2:**109-113.
56. Nakano Y, Steward N, Sekine M, Kusano T, Sano H: **A tobacco NtMET1 cDNA encoding a DNA methyltransferase: molecular characterization and abnormal phenotypes of transgenic tobacco plants.** *Plant and Cell Physiology* 2000, **41:**448-457.
57. Chapman SN, Kavanagh TA, Baulcombe DC: **Potato virus X as a vector for gene expression in plants.** *Plant J* 1992, **2:**549-557.
58. Covey SN, Al-Kaff NS, Langara A, Turner DS: **Plants combat infection by gene silencing.** *Nature* 1997, **385:**781-782.